

# POWDERED HIDE FOR RESEARCH ON TANNING MECHANISMS

by

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## ABSTRACT

The conversion of animal hides into leather, the most valuable coproduct of the US meat industry, is a multistep process that has evolved more as art form than as science. A variety of dehairing and other hide preparation processes have been adopted without an understanding of how they affect the chemical and physical properties of the resulting leather. To develop a basis for designing or selecting effective sustainable tanning agents, a suitable model system must include an evaluation of the effects of pretanning steps. A protocol for the production of powdered hide is described. Moisture, ash, total protein as collagen, hydrothermal stability, collagenase resistance, proteoglycan content and molecular weight distribution were compared for powdered hide prepared from bated and not-bated hide. This research provides a basis for assessing the effects of different process steps, and represents an initial step in the development of well-characterized model systems for comparing research from different laboratories on tanning mechanisms.

## RESUMEN

La conversión de las pieles de animales en cuero, el subproducto más valioso de la industria de la carne de los EE.UU., es un proceso de múltiples pasos que ha evolucionado más como forma de arte que como ciencia. Una variedad de depilados de la piel y otros procesos de preparación han sido adoptados sin una comprensión de cómo afectan a las propiedades químicas y físicas de la piel resultante. Para desarrollar una base para el diseño o la selección sostenible de agentes de curtido eficaces, un modelo de sistema adecuado debe incluir una evaluación de los efectos de los pasos de precurtido. Un protocolo para la producción de polvo de piel es descrito. Humedad, cenizas, proteínas totales como colágeno, estabilidad hidrotérmica, resistencia a la colagenasa, contenido de protoglicanos y la distribución del peso molecular fueron comparados con polvo de cuero preparados a partir de pieles tratadas y no tratadas con enzimas. Esta investigación proporciona una base para evaluar los efectos de las diferentes fases del proceso, y representa un primer paso en el desarrollo de sistemas modelos bien caracterizado por la comparación de los diferentes laboratorios de investigación sobre los mecanismos de curtido.

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## INTRODUCTION

Cost, yield and leather quality were the driving forces behind the development of chrome tanning. It is a tribute to the tanner that with essentially empirical approaches, the process has been refined to meet ever tightening environmental restrictions while maintaining the quality of the product. In recent years, consumer preference and environmental regulations, particularly in the European market have driven research into the development of alternative tanning methods, i.e., the quest for chrome-free leather.

An understanding of the mechanisms of tanning will provide a scientific basis for the design of economical, environmentally friendly processes to produce high quality leathers. Tanning processes are cumbersome and expensive, making model systems essential for research purposes. The first model system is often a soluble collagen model, generally using either calf skin<sup>1</sup> or rat-tail tendon<sup>2</sup> collagen to observe by spectroscopic methods, the effects of isolated tanning reactions on the conformation and conformational stability of collagen in solution. Interpretation of data obtained with these well characterized, commercially available sources of type I collagen is reasonably straight forward, although the ability of this information to add appreciably to an understanding of tanning may be limited.

A second type of laboratory scale model system for tanning studies uses pulverized bovine hide, commonly referred to as "hide powder." We and possibly others have used this term without a full appreciation of its history and meaning.<sup>3</sup> Prior to World War II, hide powder was such a major concern of leather chemists, that this journal contained numerous reports from the "Hide Powder Committee" of the Association, and records of annual meetings detailed spirited discussion on the usage and analysis of hide powder. Currently, the term 'hide powder' generally refers to a commercial product prepared by a proprietary method, available from a few sources worldwide. In a low chrome version, it is intended for the analysis of vegetable tanning agents, enzyme activity or pharmaceutical uses.<sup>4,5</sup> Commercial hide powder has been used in a variety of tanning mechanism studies.<sup>6,7</sup> Powdered hide, is not intended to compete with hide power, but rather to encourage researchers on tanning mechanisms to prepare and use a substrate that they fully understand.

Tanning has developed in a mostly empirical fashion, and the effects of individual steps in the process on the hide substance are poorly understood. Powdered hide is the model most representative of the intact hide and has the advantage that a few grams of well mixed powdered hide can be more representative of the whole hide than small piece of similar weight cut from any specific location in the hide. In this report, we describe the preparation and characterization of powdered hide for research on tanning mechanisms.

## EXPERIMENTAL

### Materials

Fresh hide was obtained from a local abattoir, cut into pieces (~15 × 40 cm) from the butt area, and used to prepare not-bated and bated powdered hide. Bating enzymes (Rohapon 6000) were from TFL, USA, Greensboro, NC. Pepsin (porcine gastric mucosa), bacterial collagenase (361 units/mg) isolated from *Clostridium histolyticum*, and Alcian Blue 8GX were obtained from Sigma-Aldrich, St. Louis, MO. Other chemicals were reagent grade.

### Preparation of Powdered Hide

The hide pieces were sulfide dehaired and relimed essentially as described by Cabeza et al.<sup>8</sup> The relimed hide was split to a thickness of 2.0 mm and washed in a drum with a 400% float for 1 h. For the not-bated powdered hide, the split was tumbled for 3 h in a 400% float adjusted to pH 7.1 by the addition of 0.5% acetic acid (unless noted otherwise, all added chemicals are based on the weight of the limed split). For the bated powdered hide, the float (400%) for the washed split was adjusted to pH 9 - 10 with 0.25% acetic acid, 0.15% bate (Rohapon 6000) was added and the mixture was tumbled for 90 minutes. After bating, the split was tumbled for 1.5 h with 400% water, the pH in the drum was adjusted to 7.1, and tumbling continued for an additional 1.5 h. From this point, the treatment of the two hide samples was identical. The pH was adjusted to 5.4 by the addition of 0.5% acetic acid to the drum and the split tumbled for 4 h, then washed in running water for 30 min and refrigerated overnight.

On the following day, the splits were tumbled twice for 2 h in 400% float pH 6.6 containing 4.35% NaCl, and then washed for 1 h with water. They were treated 3 times with 0.3 % lime in 400% float for 2 h at pH 11.5-11.7. The splits were then washed for 1 h with water, followed by tumbling overnight in 400% float containing 2.3% acetic acid and 3.2 % sodium acetate. The next day, they were tumbled at least 3 times in 400% water for 1 h until the pH of the float was the same as that of the water. Each split was then cut into strips (2 X 12 in) and stored in the freezer until they could be acetone dried.

Strips were dried with acetone, in a fume hood, in small batches. The pieces were weighed and mixed with 400% acetone, and allowed to sit overnight. This step was repeated, 5 - 7 times, until the specific gravity of the float was the same as that of acetone, and no further water could be removed. The hide pieces were then air-dried, ground in a Wiley mill and stored under refrigeration in reclosable plastic bags.

### Analyses

Moisture was determined from the weight loss when a sample was heated in an oven at 105 °C for 16 h and then cooled in a desicator.<sup>9</sup> The dried samples were further heated in a

muffle furnace at 600 °C for 2 h to determine ash content.<sup>9</sup> Total nitrogen was determined on dried samples by a semi-micro Kjeldahl method.<sup>9</sup> A factor for the conversion of nitrogen to collagen of 5.13 was calculated from the amino acid sequences of bovine type I collagen  $\alpha 1(I)$  (P02453) and  $\alpha 2(I)$  (P02465) in the UniProt data base<sup>10</sup> by the method of Sosulski and Imafidon.<sup>11</sup> All analyses were performed in triplicate.

#### Hydrothermal stability

Hydrothermal stability of powdered hide was determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.<sup>3</sup> Powdered hide samples were prepared for DSC experiments by soaking in distilled water overnight and then blotting on filter paper. Moist, blotted samples (100 - 250 mg) were weighed into ampoules that were sealed and placed in the calorimeter. The calorimeter was programmed to record heat flow as  $\mu\text{cal}/^\circ\text{C}$  while the temperature was increased from 30 °C to 130 °C at 1.5 °C/min with an equilibration period of 600 s at the start. The temperature at the peak of the calorimetry trace,  $T_p$ , was considered to be an apparent shrinkage temperature. Samples were dried, and the initial moisture content (usually ~200%) of each sample was calculated.

#### Collagenase resistance

Collagenase resistance was determined by a modification of the Mandl method.<sup>12,13</sup> Two separate samples from each treatment were analyzed in triplicate. Dry powdered hide samples were suspended in 0.05 M tris(hydroxymethyl)-methyl-2-aminoethane sulfonate (TES) buffer with 0.36 mM calcium chloride, pH 7.5, and incubated with collagenase at a ratio of 1mg collagenase to 100mg powdered hide, for 5h at 37 °C. The resulting solution was reacted with ninhydrin-citric acid and the absorbance read at 600 nm on a UV-Vis spectrophotometer. Susceptibility to collagenase is reported in terms of  $\mu\text{moles}$  of amino acid released per mg of collagen as compared with a standard curve for leucine.

#### Proteoglycan content

The core protein, decorin, was assayed on two separate samples from each treatment, by the indirect sandwich ELISA procedure developed and described in detail by Mozersky.<sup>14</sup> The sulfated glycosaminoglycan (SGAG) content was estimated as described by Mozersky et al.<sup>15</sup> The powdered hide was treated with collagenase in guanidine HCl to liberate the SGAG which were then assayed with Alcian Blue.<sup>15</sup>

#### Electrophoresis

Powdered hide samples were digested with pepsin (1mg pepsin + 100 mg powdered hide)/ml overnight at 4 °C.<sup>16</sup> The digested samples were centrifuged at room temperature for 0.5 h at 1000 RPM, and the supernatant, was dialyzed, in Slide-A-Lyzer dialysis cassettes (10,000 MWCO) 3-12 ml capacity (Thermo Scientific, Rockford, IL) for two days against four changes of 0.5 M acetic acid, and then lyophilized. Solubilized powdered hide samples were prepared for SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) analysis as described by Taylor et al.<sup>17</sup> Separation was achieved on a PhastGel System (GE Life Sciences, Piscataway, NJ) using an extended protocol to assure that intact protocollagen would move into the gel. A broad range SDS-Standard (Bio-Rad, Hercules, CA) containing nine proteins ranging in size from 6,500 to 200,000 Daltons, and type I insoluble collagen (Sigma, St. Louis) were included as controls.

## RESULTS AND DISCUSSION

Results of moisture, ash, and protein analyses of freshly prepared powdered hide from not-bated and bated hide are summarized in Table I. Triplicate results on a single sample are in excellent agreement, and those for separate samples from the same treatment are in reasonable agreement for a natural material. Powdered hide from not-bated hides retained more moisture (9 - 14%) than did that from bated hide (8 - 8.6%) when they were air dried, most probably because more

**TABLE I**  
**Analysis of Powdered Hide**

Sample <sup>a</sup>	Moisture, %	Ash <sup>b</sup> , %	Protein <sup>b</sup> , %	$T_p$ <sup>c</sup> , °C
NB-1	14.2±0.1	0.34±0.03	91.9±1.7	66.4±0.4
NB-2	9.2±0.1	0.40±0.05	95.6±1.9	63.5±0.5
B-1	8.6±0.2	0.29±0.06	95.5±0.9	64.9±0.7
B-2	8.0±0.1	0.44±0.09	90.4±1.6	64.4±0.3

<sup>a</sup>Two separate samples from each treatment were analyzed in triplicate, NB - not-bated, B - bated.

<sup>b</sup>On a dry weight basis.

<sup>c</sup>On a wet sample basis, moisture content approximately 200% of dry sample weight.

surface area was exposed after bating. Ash content on a moisture free basis was uniform (0.3 - 0.4%), and lower than the 0.5 - 1% reported for commercial hide powders.<sup>4,5</sup> Protein, calculated as type I collagen from total nitrogen determined on dry material was 90 to 96% in both the bated and not-bated samples.

#### Hydrothermal stability

The apparent shrinkage temperature,  $64.8 \pm 1.2$  °C, determined by DSC for our freshly prepared powdered hide from bated or not-bated hides was significantly greater than the 60 °C determined by the same protocol for the material used earlier in our genipin studies.<sup>3</sup> That material had been stored for several years and may have deteriorated. A typical shrinkage temperature for raw-hide measured in a shrink-temperature apparatus as described by Fein et al.<sup>18</sup> is about 66 °C.<sup>19</sup> Factors that may contribute to differences between the shrinkage temperatures determined for a strip of hide and the apparent shrinkage temperatures determined for powdered hide include the exposed surface area, the degree of hydration and the rate of heating. DSC is the most widely used research tool for monitoring thermal stability of collagen in powdered hide. The typical DSC configuration, a small volume (<100  $\mu$ L) sample container and the need for a rapid scan to provide adequate sensitivity, may lead to artificially high temperature values.<sup>20</sup> Because samples for the DSC configuration used in this research are well hydrated, the apparent slight decrease in hydrothermal stability of the powdered hide relative to a piece of hide may be related to the heating rate of 1.5 °C per min in these experiments that is slower than the 5 °C per min used in more conventional DSC experiments.<sup>19</sup>

#### Collagenase resistance

Leucine, an amino acid with no reactive or chromophoric sidechain group, is frequently used as a standard in assays that measure changes in the numbers of primary amino groups as a result of proteolysis or crosslinking. Interestingly, the available primary amine groups prior to collagenase treatment were equivalent to  $7.15 \pm 1.3$   $\mu$ mol Leu  $\text{mg}^{-1}$  for powdered hide prepared from either bated or not-bated hide. Thus, proteinaceous matter digested by the bating enzymes was washed out of the powdered hide during processing, and the collagen was left essentially intact. Not-bated and bated powdered hide were susceptible to collagenase digestion to different degrees. The 5 h digestion with collagenase liberated primary amino groups equivalent to  $16.5 \pm 6.7$   $\mu$ mol Leu  $\text{mg}^{-1}$  from not-bated powdered hide and  $26.9 \pm 4.8$   $\mu$ mol Leu  $\text{mg}^{-1}$  from bated powdered hide. Although the results were more variable after collagenase digestion, the difference between the treatments is notable. As one might expect, the bating process, by removing noncollagenous protein from the hide substance exposes more of the collagen to attack by collagenase.

#### Proteoglycans

Different dehairing methods, and other pretanning steps may have consequences for the characteristics of the hide as it enters the beamhouse. The decorin core protein remaining in the not-bated powdered hide was  $0.77 \pm 0.15$  mg/g, and  $0.59 \pm 0.06$  mg/g in the bated powdered hide, representing a 22% decrease in decorin protein when the hide had been bated. The SGAG content in the not-bated powdered hide was  $0.19 \pm 0.03$  mg/g and  $0.22 \pm 0.03$  mg/g in the bated hide. The amounts of SGAG extracted from the two treatments are essentially the same. Of interest, is the difference in the ratio of SGAG to core protein, which was 0.25 for not-bated powdered hide, and 0.38 for bated powdered hide. The reasons for this difference are not entirely clear, but they suggest that the bating process is more effective at hydrolyzing the core protein than at removing protein fragments that bind SGAG to collagen.

#### Electrophoresis

Samples of pepsin treated not-bated and bated powdered hide, and insoluble collagen gave essentially identical SDS-PAGE patterns, Figure 1. The patterns are similar to those reported by Lin and Liu,<sup>21</sup> with major bands for the collagen  $\beta$ -chain near 200 kDa, and the  $\alpha 1$ - and  $\alpha 2$ - chains slightly above the 116 kDa marker. Very faint bands can be seen in the 35 - 45 kDa region, representing smaller protein components of the extracellular matrix, possibly including the residual decorin core protein at about 45 kDa and pepsin at 35 kDa. A fraction of the material was in large aggregates that did not penetrate the gel, but remained in the lower density stacking gel. Only minor amounts of low molecular fragments were seen in any of the patterns.

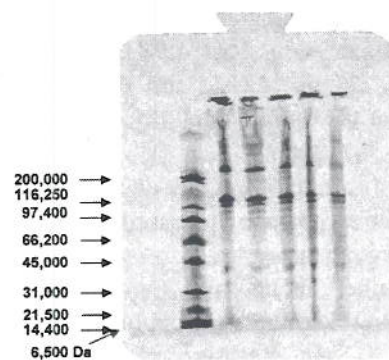


Figure 1. - SDS-PAGE gel, 4 - 15% gradient. Shown from left to right: broad range molecular weight standard, two samples of pepsin solubilized powdered hide from bated hide, two from not-bated hide, and a commercial insoluble collagen.

## CONCLUSIONS

Tanning processes are continually evolving in response to environmental, economic and legislative pressures. Changes in a process in response to one or more of these pressures may have unanticipated effects further into the process or on the final product. An understanding of the mechanisms of tanning depends on knowledge of the effects on the hide substance of beamhouse processes that precede the tanning step. To develop a basis for designing or selecting effective sustainable tanning agents to produce leathers with desirable physical and subjective properties, a suitable model system must include an evaluation of the effects of pretanning steps. The adoption of a relatively standard model system, or protocol for describing the model system, for research on tanning mechanisms at an intermediate level between soluble collagen and intact hides will facilitate collaborations and information exchanges among laboratories around the world. This study, while not definitive, represents a start in that direction.

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